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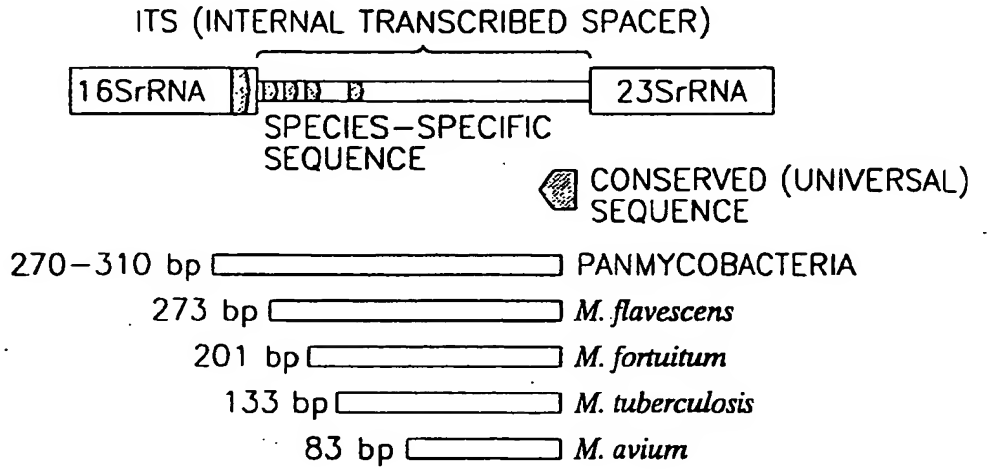

(57) Abstract: A new multiplex polymerase chain reaction (PCR) method and a multiplex PCR kit and oligonucleotides with which *Mycobacteriay* species can specifically and rapidly be identified based on the multiplex PCR method are provided. For the multiplex PCR method, at least two target genes are simultaneously amplified in a single tube through a single reaction using at least one oligonucleotide having a common sequence for the at least two target genes to be amplified, as a fixed primer(s), and at least two oligonucleotides each having gene-specific sequences as specific primers. The multiplex PCR kit uses an oligonucleotide primer having a conserved sequence of *Mycobacteria* as a fixed primer, and at least two oligonucleotide primers, which are specific to *Mycobacteria* species, as specific primers. Using this multiplex PCR kit, at least two *Mycobacteria* species can be identified through a single PCR. Thus, the multiplex PCR kit can be effectively applied in diagnosing *M. tuberculosis* and detecting non-*tuberculosis Mycobacteria* at low costs.

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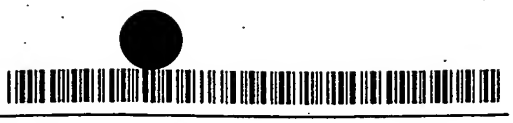

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MULTIPLEX PCR METHOD AND KIT AND OLIGONUCLEOTIDES FOR DETECTION AND IDENTIFICATION OF MYCOBACTERIA USING THE MULTIPLEX PCR METHOD

Technical Field

The present invention relates to a new multiplex polymerase chain reaction (PCR) method and a multiplex PCR kit and oligonucleotides for rapid and specific identification of *Mycobacteria* species, and more particularly, to a multiplex PCR method capable of detecting *Mycobacteria* genus and identifying *M. tuberculosis* (MTB) and non-*tuberculosis Mycobacteria* (NTM) species thereof in a single PCR using genus-specific and species-specific primers derived from the internal transcribed spacer (ITS) sequences of *Mycobacteria*.

Background Art

Mycobacteria are major pathogens causing human diseases. In the world, 800 million people are infected every year by *Mycobacterium tuberculosis* and 300 million people among them die from the infection (Raviglione, M. C., D. E. Snider, and A. Kochi, "Global epidemiology of tuberculosis, morbidity and mortality of a worldwide epidemic," *JAMA*, 271:220-226, 1995). Recently, as the number of peoples suffering from AIDS rapidly increases, infections caused by non-*tuberculosis Mycobacteria* (NTM) are gradually increasing (Barnes, P., A. B. Bloch, P. T. Davidson, and D. E. Snider, "Jr. Tuberculosis in patients with immunodeficiency virus infection," *N. Engl. J. Med.*, 324:1644-1650, 1991). For this reason, there is an urgent need to develop a method for rapidly and efficiently identifying non-*tuberculosis Mycobacteria* as well as *Mycobacterium tuberculosis* and diagnosing infections caused by the same.

Most conventional methods for identifying and classifying bacteria are based on the morphological, biochemical, and growth characteristics of bacteria. These conventional methods are very tedious and complicated to conduct and take much time. Due to these problems, use of a simple and rapid identification method using a gene as a target sequence becomes more common. For an emerging gene-based

identification method, genus-specific or species-specific PCR primers or nucleotide probes are applied to a gene of interest.

Under this situation, pedigree analysis on *Mycobacteria*, which would provide the base for species identification, has been conducted by comparison of 16S rRNA or its nucleotide sequences. This is based on the fact that the16S rRNA gene has the conserved but polymorphic sequences for *Mycobacteria* species identification (Stahl, D. A., and J. W. Urbane, "The division between rapid and slow-growing species corresponds to natural relationships among the *Mycobacteria*," *J. Bacteriol.*, 172:116-124 (1990); Rogall, T., J. Wolters, T. Flohr, and E. C. Bottger, "Toward a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*," *Int. J. Syst. Bacteriol.*, 40:323-30 (1990b); Rogall T., T. Flohr, and E. C. Bottger, "Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA," *J. Gen. Microbiol.*, 136 (Pt9):1915-1920 (1990a)). However, since the 16S rRNA gene has similar nucleotide sequences among some species, there is a limitation in identifying species to a certain extent (Fox, G. E., J. D. and P. J. Jurtshum, "How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity," *Int. J. Syst. Bacteriol.* 42:166-170 (1992)).

Also, IS6110 insertion element has multiple copy numbers in TB complexes of *M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. microti*, and thus a PCR probe method using IS6110 as a target sequence is used. However, *Mycobacterium tuberculosis* which do not have the IS6110 insertion element have been reported and thus may yield pseudo-negative test results (Yuen L. K., B. C. Ross, K. M. Jackson, and B. Dwyer, "Characterization of *Mycobacterium tuberculosis* strains from Vietnamese patients by Southern blot hybridization," *J. Clin. Miclobiol.*, 31:1615-1618 (1993)). Although primers capable of amplifying the IS6110 insertion element and a detection kit therefor are commercially available as TB-PCR and TB Detection Kit (from Bioneer Co., Korea), application of these primers is restricted to certain species and the PCR kit can only detect the presence of TB complex.

As infections caused by non-*tuberculosis Mycobacteria* (NTM), and particularly by unidentified new species of NTM, are increasing, there is a need for the development of a new species-specific

sequence-based identification and diagnosis method so as to accurately identify disease causing species to prevent and remedy infections caused by new species of NTM.

Disclosure of the Invention

To solve the above-described problems, it is a first object of the present invention to provide a multiplex polymerase chain reaction (PCR) method capable of amplifying at least two target genes in a single PCR using a minimum number of primers.

It is a second object of the present invention to provide a multiplex PCR kit for use in the new PCR method in which detection of *Mycobacteria* genus and identification of *M. tuberculosis* (MTB) and non-*tuberculosis Mycobacteria* (NTM) species can be achieved at the same time through a single PCR using a minimum number of primers.

It is a third object of the present invention to provide oligonucleotide primers for use in a new multiplex PCR method in which detection of *Mycobacteria* genus and identification of *M. tuberculosis* and non-*tuberculosis Mycobacteria* species can be achieved at the same time.

The first object of the present invention is achieved by a PCR method for simultaneously amplifying at least two target genes in a single tube through a single reaction using at least one oligonucleotide having a common sequence for the at least two target genes to be amplified, as a fixed primer(s), and at least two oligonucleotides each having gene-specific sequences as specific primers. It is preferable that the number of fixed primers is one and the number of specific primers is an integer from 2 to 6.

The second object of the present invention is achieved by a multiplex PCR kit comprising: at least one oligonucleotide having a common sequence for at least two target genes to be amplified, as a fixed primer(s); and at least two oligonucleotides each having species-specific sequences as specific primers, wherein the fixed primer(s) and the specific primers are simultaneously reacted in a single tube.

It is preferable that for the multiplex PCR kit, the fixed primer is an oligonucleotide primer having a common sequence for *Mycobacteria*

genus and the specific primers are oligonucleotide primers each having *Mycobacteria* species-specific sequences, such that identifying *Mycobacteria* and diagnosing infections caused by *Mycobacteria* are possible. More preferably, the fixed primer comprises the oligonucleotide having SEQ ID NO. 1 and the specific primers comprise at least two oligonucleotides having SEQ ID NOs. 2 through 6.

For example, when the fixed primer comprises the oligonucleotide having SEQ ID NO. 1 and the specific primers comprise the oligonucleotides having SEQ ID NOs. 2 and 3, detection of *Mycobacteria* genus and identification of *M. tuberculosis* can be achieved at the same time. When the specific primers comprise the oligonucleotides having SEQ ID NOs. 2 and 6, detection of *Mycobacteria* genus and identification of *M. avium* can be achieved at the same time. When the specific primers comprise the oligonucleotides having SEQ ID NOs. 2, 3 and 6, detection of *Mycobacteria* genus and identification of *M. tuberculosis* or *M. avium* can be achieved at the same time.

The third object of the present invention is achieved by an oligonucleotide having SEQ ID NO. 1 for use in detecting *Mycobacteria* genus, an oligonucleotide having SEQ ID NO. 3 for use in identifying *Mycobacterium tuberculosis*, an oligonucleotide having SEQ ID NO. 4 for use in identifying *Mycobacterium fortuitum* as a non-*tuberculosis Mycobacteria*, an oligonucleotide having SEQ ID NO. 5 for use in identifying *Mycobacterium flavescens* as a non-*tuberculosis Mycobacteria*, and an oligonucleotide having SEQ NO. 6 for use in identifying *Mycobacterium avium* as a non-*tuberculosis Mycobacteria*.

Brief Description of the Drawings

FIG. 1 is a schematic representation of locations of the oligonucleotide primers used in a multiplex polymerase chain reaction (PCR) method according to the present invention, and a map of PCR amplification products;

FIG. 2 shows an electrophoresis image taken after a PCR using genus-specific primers ITSF and MYC6 for Panmycobacteria;

FIG. 3 shows an electrophoresis image taken after a PCR using species-specific primers MTB10, FOR12, FLA9, and MAC5 for *Mycobacteria*;

FIG. 4 shows an electrophoresis image taken after a multiplex PCR according to the present invention on a *M. tuberculosis* (MTB) and six non-*tuberculosis Mycobacteria* (NTM) using primers ITSF, MTB10, FOR12, FLA9, MAC5, and MYC6; and

FIG. 5 is a schematic representation of the electrophoresis image of FIG. 4.

Best mode for carrying out the Invention

According to the present invention, a multiplex polymerase chain reaction (PCR) method is developed for identifying *Mycobacteria* and diagnosing infections caused by the same, in which nucleotide sequences of an internal transcribed spacer (ITS) region between the 16S rRNA and 23S rRNA genes, which has genus- and species-specific sequences for *Mycobacteria*, are used as a target sequence.

In other words, from the ITS region between the 16S rRNA and 23S rRNA genes, which contain both conserved and polymorphic sequences for *Mycobacteria*, a genus-specific primer having the conserved sequence and species-specific primers having the polymorphic sequences are derived. One conserved sequence region is used as a common reverse primer, and four species-specific primers combined with the one genus-specific primer are used as forward primers to obtain PCR products having different sizes. As a result, the *Mycobacteria* genus can be detected and at the same time *M. tuberculosis* (MTB) and non-*tuberculosis Mycobacteria* (NTM) species can be identified.

These primers are designed such that PCR products obtained using the primers have different sizes for easy discrimination thereof by gel electrophoresis. All six primers are simultaneously applied to a single PCR to detect the *Mycobacteria* genus and identify *M. tuberculosis* and non-*tuberculosis Mycobacteria* species through the single PCR.

In general, one primer pair is used for detection of one target strain. Thus, 5 primer pairs, i.e., 10 primers, are required to detect five targets through five separate PCRs. Although the number of reactions required can be reduced by multiplex PCR, the number of primers required cannot be reduced even for the multiplex PCR. In this aspect,

the present inventor has researched to reduce the number of primers required to a minimum number and further develop primers having the same reaction conditions, which allows the primers to be simultaneously applied to the same PCR. As a result, detecting the *Mycobacteria* genus and identifying MTB and NTM species through a single PCR using only six primers can be realized.

The PCR primers according to the present invention are synthesized from an ITS sequence of *Mycobacteria*. The multiplex PCR method according to the present invention for detecting the *Mycobacteria* genus and at the same time identifying MTB and NTM species through a single reaction uses six primers including species-specific forward primers and one common genus-specific reverse primer. When designing the primers, many restrictions are applied, such as ratios of A, G, C, and T of the primers, preventing formation of a dimer between the primers, and prohibiting three or more repetitions of the same sequence. In addition, PCR conditions, such as concentrations of template DNA, primers, dNTP, and Mg^{2+}, reaction temperature, and reaction time, should be appropriate.

For the multiplex PCR method according to the present invention for detecting the *Mycobacteria* genus and identifying both MTB and NTM species through a single reaction by combination of appropriate primers, the reaction conditions should be further restricted when designing the primers. In addition, the primers should be designed such that the amplification products have different sizes and can be distinguished from one another on a gel after the PCR.

Six primers for use in detecting the *Mycobacteria* genus and identifying MTB and NTM species according to the present invention are designed to be appropriate for the multiplex PCR method. All six primers can be simultaneously applied to a single PCR, and thus the *Mycobacteria* genus can be detected and both MTB and NTM species can be identified through a single PCR with high sensitivity. The PCR primers according to the present invention and the sizes of PCR products obtained using the primers are shown in Table 1.

Table 1

Strain	Forward Primer	Reverse Primer	Size of Amplification

			Product
Mycobacteria genus	ITSF (SEQ ID NO. 2)	MYC6 (SEQ ID NO. 1)	varies slightly depending on species
M. tuberculosis	MTB10 (SEQ ID NO. 3)		139 bp
M. fortuitum	FOR12 (SEQ ID NO. 4)		206 bp
M. flavescens	FLA9 (SEQ ID NO. 5)		277 bp
M. avium	MAC5 (SEQ ID NO. 6)		78 bp

The MYC6 (SEQ ID NO. 1) is the conversed sequence of *Mycobacteria* genus and its sense or antisense sequence can be used as a common reverse primer (fixed primer) in the present invention. The ITSF (SEQ ID NO. 2) has the conserved sequence of the 16S RNA region, however is used as a specific primer in the present invention because it is a forward primer used for the detection of *Mycobacteria*. The MTB10 (SEQ ID NO. 3), FOR12 (SEQ ID NO. 4), FLA9 (SEQ ID NO. 5) and MAC5 (SEQ ID NO. 6) are the specific sequences of MTB or NTM species and their sense or antisense sequences can be used as forward primers (specific primers) in the present invention.

FIG. 1 is a schematic representation of the map of amplification products from the multiplex PCR according to the present invention and locations of the primers used for the amplification.

The present invention will be described in greater detail by means of the following examples. The following examples are for illustrative purposes and are not intended to limit the scope of the invention.

Example 1: Incubation of Standard *Mycobacteria* Strains and Isolation of Genomic DNA

Standard strains of *Mycobacteria* were obtained from the Korean Collection for Type Culture (KCTC) and the American Type Culture Collection (ATCC). The DNA of these strains were isolated using InstaGene matrix (Bio-Rad Co.).

200 μl InstaGene matrix was put into a 1.5 ml tube. A target strain was incubated in a solid medium (Ogawa plate), one scrapping of the strain layer was removed with an inoculating loop and suspended in the InstaGene Matrix contained in the tube. The suspension was reacted at 56°C for 30 minutes and mixed thoroughly for 10 seconds. The mixture was heated at 100°C for 8 minutes and mixed thoroughly for 10 seconds. The mixture was centrifuged at 12,000 rpm for 3 minutes and the supernatant was collected as a template DNA for PCR.

The standard strains used were:

M. tuberculosis	H37Rv (ATCC 27294)
M. fortuitum	(ATCC 6841)
M. flavescens	(ATCC 14474)
M. avium	(ATCC 25291)
M. kansasii	(ATCC 12478)
M. chelonae	(ATCC 35752)
M. szulgai	(ATCC 35799)

Example 2: Preparation of Primers and Probes for use in PCR

1) Preparation of primers and probes for detection of *Mycobacteria* gene

As primers capable of amplifying only *Mycobacteria* gene, not other pathogenic microorganisms, conserved sequences existing in all *Mycobacteria* were chosen. ITSF (SEQ ID NO. 2) as a forward primer and MYC6 (SEQ ID NO. 1) as a reverse primer were prepared by well-known methods.

2) Preparation of Primers and Probes for identification of MTB species

As primers capable of specifically amplifying MTB, MTB 10 (SEQ ID NO. 3) as a forward primer and MYC6 (SEQ ID NO. 1) as a reverse primer were prepared from the sequences of the ITS region of MTB.

3) Preparation of Primers and Probes for identification of NTM species

As NTM-specific primers capable of amplifying each NTM species into amplification products having different sizes for easy separation on a gel after the reaction, FOR12 (SEQ ID NO. 4), FLA9 (SEQ ID NO. 5), and MAC5 (SEQ ID NO. 6) as forward primers, and MYC6 (SEQ ID. NO. 1) as a reverse primer were prepared from the sequence of the ITS region of NTM.

Example 3: Primer Specificity Test

Prior to application of the multiplex PCR to the standard *Mycobacteria* strains, separate PCRs were carried out on various strains including four target strains using each of the prepared primers. After sufficient denaturation at 94°C for 5 minutes, 40 cycles of amplification at 94°C for 1 minute, at 64°C for 1 minutes, and at 72°C for 1 minute were carried out and followed by a single final extension at 72°C for 10 minutes. After the reaction, the reaction products were analyzed by electrophoresis on a 3% agarose gel.

FIG. 2 is an electrophoresis image taken after the PCR performed using primers ITSF and MYC6 derived from the *Mycobacteria* genus-specific sequence. In FIG. 2, lane M represents an index of molecular weight of 100 basepair (bp), lane C represents distilled water used as a negative control, lane 1 represents *M. tuberculosis*, lane 2 represents *M. flavescens*, lane 3 represents *M. avium*, lane 4 represents *M. fortuitum*, lane 5 represents *M. kansasii*, lane 6 represents *M. chelonae*, and lane 7 represents *M. szulgai*. It is apparent from FIG. 2 that only *Mycobacterium* microorganisms are amplified through the PCR using the primers ITSF and MYC6.

FIG. 3 is an electrophoresis image taken after a PCR performed using species-specific primers MTB10, FOR12, FLA9, and MAC 5 derived from *Mycobacteria* species-specific sequences. In FIG. 3, images A, B, C, and D are for *M.tuberculosis*, *M. flavescene*, *M. avium*, and *M. fortuitum*, respectively. Lane M represents an index of molecular weight of 100 bp, lane C represents distilled water used as a negative control, lane 1 represents *M. tuberculosis*, lane 2 represents *M. flavescens*, lane 3 represents *M. avium*, lane 4 represents *M. fortuitum*, lane 5 represents *M. kansasii*, lane 6 represents *M. chelonae*, and lane 7 represents *M. szulgai*. As shown in FIG. 3, it is apparent that

amplification of *M. tuberculosis* (image A) occurs only in lane 1, *M. flavescens* (image B) only in lane 2, *M. avium* (image C) only in lane 3, and *M. fortuitum* (image D) only in lane 4.

Example 4: Multiplex PCR Method for Standard Strains

A multiplex PCR method was carried out for MTB and six NTM species using primers ITSF, MTB10, FOR12, FLA9, MAC5, and MYC6 according to the present invention. The reaction mixture contained 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100, 2.5 mM each dNTP (dATP, dGTP, dTTP, and dCTP), 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, a PCR enhancer, 10 pmols of each species-specific primer, and 30 pmols of the common reverse primer. After sufficient denaturation at 94°C for 5 minutes, 40 cycles of amplification at 94°C for 1 minute, at 64°C for 1 minutes, and at 72°C for 1 minute were carried out and followed by a single final extension at 72°C for 10 minutes. After the reaction, the reaction products were analyzed by electrophoresis on a 3% agarose gel.

FIG. 4 is an electrophoresis image taken after the PCR performed for MTB and six NTM species using the primers ITSF, MTB10, FOR12, FLA9, MAC5, and MYC6. In FIG. 4, lane M represents an index of molecular weight of 100 bp, lane C represents distilled water used as a negative control, lane 1 represents *M. tuberculosis*, lane 2 represents *M. flavescens*, lane 3 represents *M. avium*, lane 4 represents *M. fortuitum*, lane 5 represents *M. kansasii*, lane 6 preresents *M. chelonae*, and lane 7 represents *M. szulgai*. As shown in FIG. 4, *Mycobacteria* genus-specific amplification occurs in all lanes 1 through 7. Amplification of one MTB occurs in lane 1, *M. flavescens* in lane 2, *M. avium* in lane 3, and *M. fortuitum* in lane 4. For three *NTMs*, *M. kansasii*, *M. chelonae*, and *M. szulgai*, only *Mycobacteria* genus-specific amplification not specific to the species occurs.

FIG. 5 is a schematic representation of the electrophoresis image of FIG. 4. In FIG. 5, bands 1, 3, 5, 7, 9, 10 and 11 represent the PCR amplification products by the *Mycobacteria* genus-specific primer pair ITSF-MYC6, which is common for the species, band 2 represents the PCR amplification product by *M. tuberculosis*-specific primer pair MTB10-MYC6, band 4 represents the PCR amplification product by *M.*

flavescence-specific primer pair FLA9-MYC6, band 6 represents the PCR amplification product by *M. avium*-specific primer pair MAC5-MYC6, and band 8 represents *M. fortuitum*-specific primer pair FOR12-MYC6.

As shown in FIGS. 4 and 5, it is evident that species-specific amplification can occur in a single tube with a plurality of mixed primers.

The species-specific primers according to the present invention have the same PCR conditions and thus they can be simultaneously applied to a single PCR. Thus, by the multiplex PCR according to the present invention which uses the six primers at the same time, MTB and NTMs can be identified through a single multiplex PCR.

Although the present invention is described with reference to the penta-multiplex PCR method in which a plurality of bacteria species can be identified through a single PCR in one test tube using one fixed primer which is common for the genus, and five genus- and species-specific primers, the present invention can be applied to any type of multiplex PCR including di-, tri-, tetra-, and hexa-multiplex PCRs within the scope of the present invention.

Industrial Applicability

As described above, in the multiplex PCR method according to the present invention, the *Mycobacteria* genus and four kinds of MTB and NTM species can be identified at the same time through a single PCR in one test tube using a minimum number of primers. The multiplex PCR method according to the present invention ensures rapid and accurate diagnosis at low costs without conducting other biochemical tests. The multiplex PCR method according to the present invention can identify at least two species through a single method; and thus it can be efficiently applied for the diagnosis of tuberculosis. In addition, the multiplex PCR method according to the present invention is effective in detecting NTM at low costs.

What is claimed is:

1. A multiplex polymerase chain reaction (PCR) method for simultaneously amplifying at least two target genes in a single tube through a single reaction using at least one oligonucleotide having a common sequence for the at least two target genes to be amplified, as a fixed primer(s), and at least two oligonucleotides each having gene-specific sequences as specific primers.

2. The multiplex PCR method of claim 1, wherein the number of fixed primers is one and the number of specific primers is an integer from 2 to 6.

3. A multiplex polymerase chain reaction (PCR) kit comprising:

at least one oligonucleotide having a common sequence for at least two target genes to be amplified, as a fixed primer(s); and

at least two oligonucleotides each having gene-specific sequences as specific primers,

wherein the fixed primer(s) and the specific primers are simultaneously reacted in a single tube.

4. The multiplex PCR kit of claim 3 for identifying *Mycobacteria* and diagnosing infections caused by *Mycobacteria*, wherein the fixed primer is an oligonucleotide primer having a common sequence for *Mycobacteria* genus and the specific primers are oligonucleotide primers each having *Mycobacteria* species-specific sequences.

5. The multiplex PCR kit of claim 3 for identifying *Mycobacteria* and diagnosing infections caused by *Mycobacteria*, wherein the fixed primer is a genus-specific primer having a conserved sequence derived from an internal transcribed spacer (ITS) between 16S rRNA and 23S rRNA of a *Mycobacteria* genus and the specific primers are species-specific primers having a polymorphic sequence derived from the ITS between 16S rRNA and 23S rRNA.

6. The multiplex PCR kit of claim 3 for identifying *Mycobacteria* and diagnosing infections caused by *Mycobacteria*, wherein the fixed primer comprises the oligonucleotide having SEQ ID NO. 1 and the specific primers comprise at least two oligonucleotides having SEQ ID NOs. 2 through 6.

7. The multiplex PCR kit of claim 6 for identifying *Mycobacteria* and diagnosing infections caused by *Mycobacteria*, wherein the fixed primer comprises the oligonucleotide having SEQ ID NO. 1 and the specific primers comprise the oligonucleotides having SEQ ID NOs. 2 and 3.

8. The multiplex PCR kit of claim 6 for identifying *Mycobacteria* and diagnosing infections caused by *Mycobacteria*, wherein the fixed primer comprises the oligonucleotide having SEQ ID NO. 1 and the specific primers comprise the oligonucleotides having SEQ ID NOs. 2 and 6.

9. The multiplex PCR kit of claim 6 for identifying *Mycobacteria* and diagnosing infections caused by *Mycobacteria*, wherein the fixed primer comprises the oligonucleotide having SEQ ID NO. 1 and the specific primers comprise the oligonucleotides having SEQ ID NOs. 2, 3 and 6.

10. An oligonucleotide having SEQ ID NO. 1 for use in detecting *Mycobacteria*.

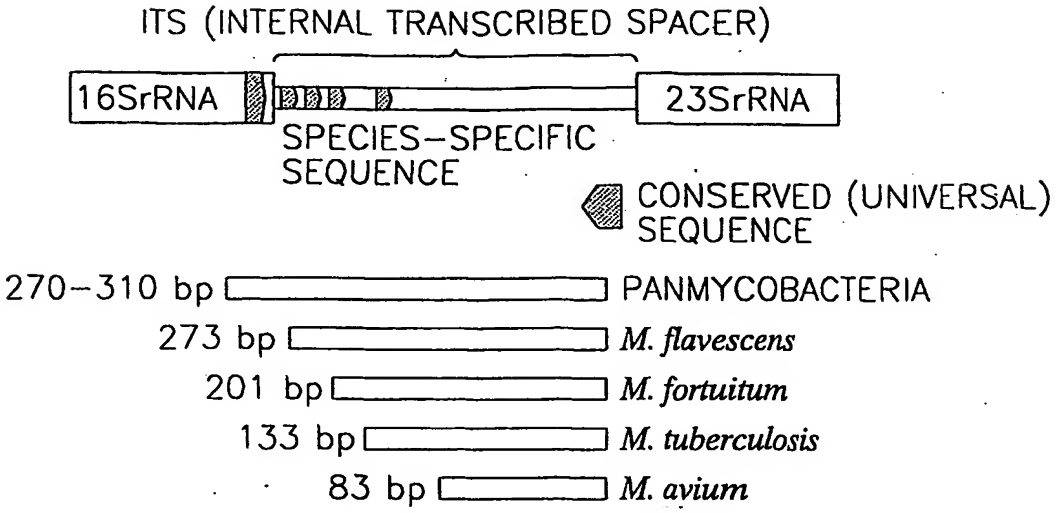
11. An oligonucleotide having SEQ ID NO. 3 for use in identifying *Mycobacterium tuberculosis*.

12. An oligonucleotide having SEQ ID NO. 4 for use in identifying *Mycobacterium fortuitum* as a non-*tuberculosis Mycobacteria*.

13. An oligonucleotide having SEQ ID NO. 5 for use in identifying *Mycobacterium flavescens* as a non-*tuberculosis Mycobacteria*.

14. An oligonucleotide having SEQ NO. 6 for use in identifying *Mycobacterium avium* as a non-*tuberculosis Mycobacteria*.

FIG. 1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23SrRNA
SPECIES-SPECIFIC SEQUENCE
CONSERVED (UNIVERSAL) SEQUENCE
270-310 bp
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273 bp
M. flavescens
201 bp
M. fortuitum
133 bp
M. tuberculosis
83 bp
M. avium

FIG. 2

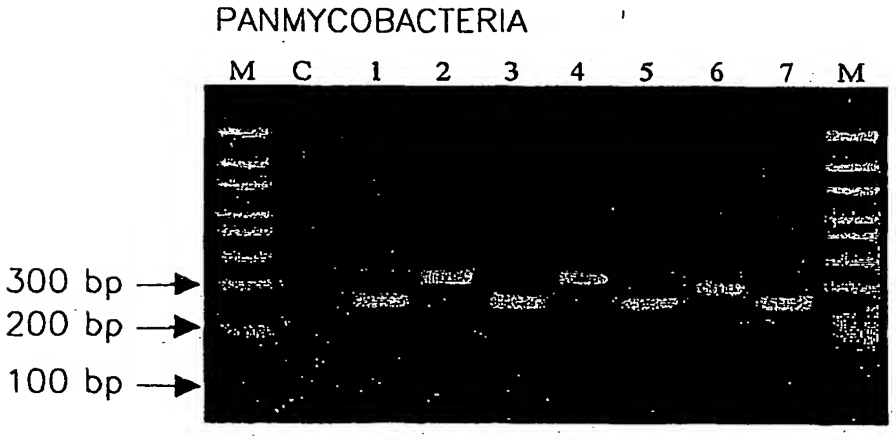

PANMYCOBACTERIA
M C 1 2 3 4 5 6 7 M
300 bp
200 bp
100 bp

FIG. 3

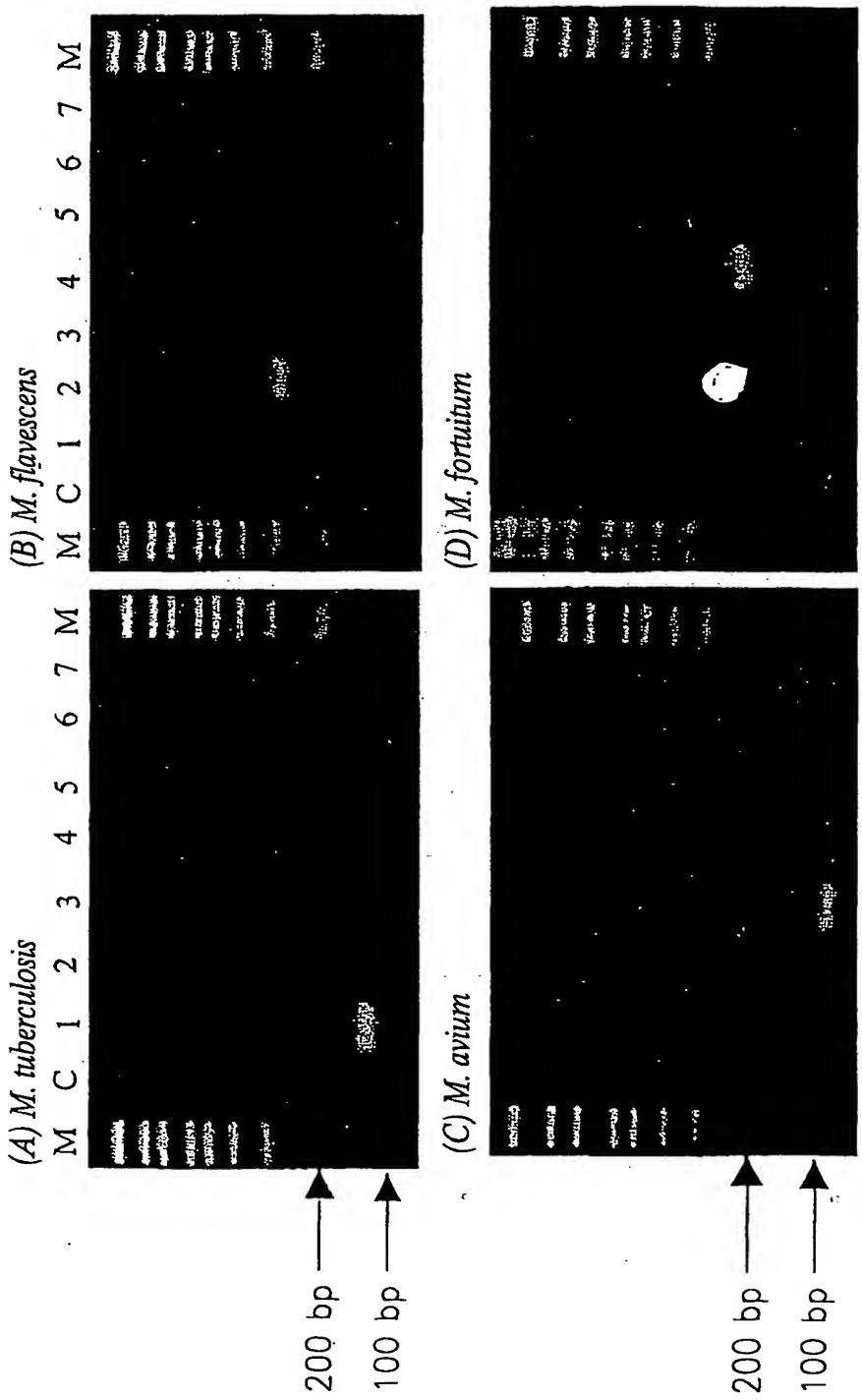

(A) M. tuberculosis
M C 1 2 3 4 5 6 7 M
(B) M. flavescens
M C 1 2 3 4 5 6 7 M
(C) M. avium
(D) M. fortuitum
200 bp
100 bp
200 bp
100 bp

FIG. 4

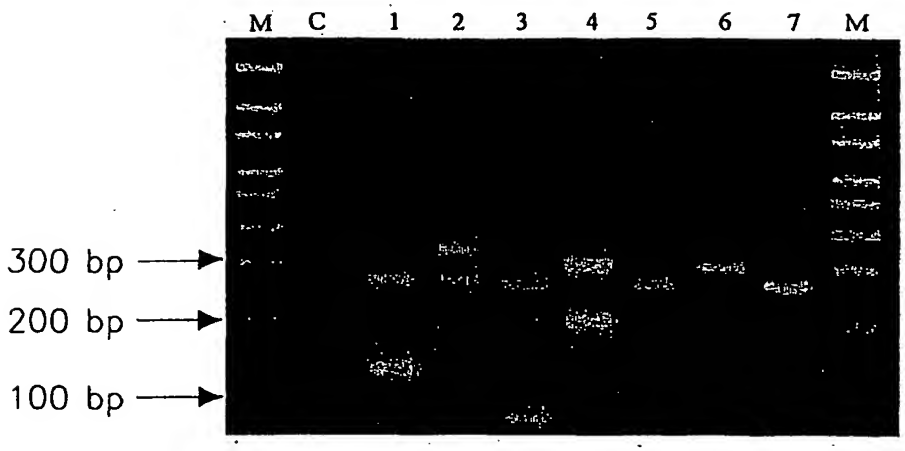
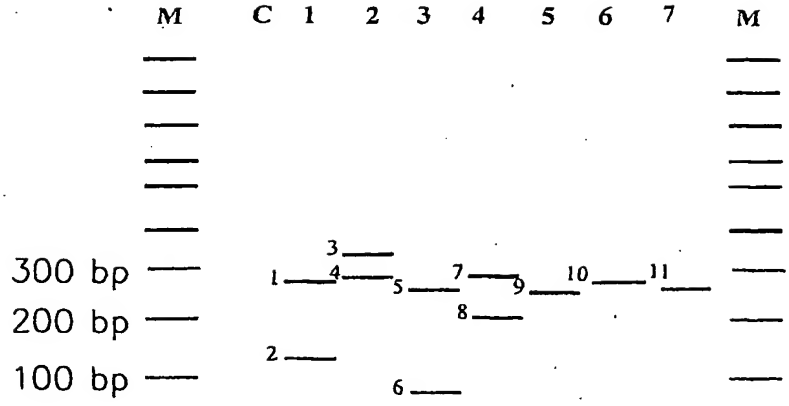

M C 1 2 3 4 5 6 7 M
300 bp
200 bp
100 bp

FIG. 5


M C 1 2 3 4 5 6 7 M
300 bp
200 bp
100 bp
1
2
3
4
5
6
7
8
9
10
11

SEQUENCE LISTING

<110> KIM, Jeong Joon; SJ HIGHTECH Co., Ltd.
KIM, Cheol Min
PARK, Hee Kyung

<120> MULTIPLEX PCR, KIT AND OLIGONUCLEOTIDE FOR DETECTION AND IDENTIFICATION OF MYCOBACTERIA USING MULTIPLEX PCR

<130> ppa90809

<160> 6

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<400> 6
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01385

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC7 C12Q 1/68

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)

Medline "multiplex PCR" and simultan*"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG et al. 'A multiplex PCR for Massachusetts andArkansas serotypes of infectious bronchitis virus' In:Mol Cel Probes, 1999, Vol.13, No.(1), p1-7	1 - 3
X	Hendolin PH et al. 'Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions' In: J Clin Microbiol, 1997, Vol.35, No.11, p2854-2858	1 - 3
A	WO 00/73436 A1 (SJ HIGHTECH CO.) 16 MAY 2000	1 - 14
A	GENBANK Accession No. AF144326 'Mycobacterium fortuitum 16S-23S intergenic spacer region, complete sequence'	12
A	GENBANK Accession No. AF191086 'Mycobacterium flavescens internal transcribed spacer, complete sequence'	13

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 DECEMBER 2001 (19.12.2001)	20 DECEMBER 2001 (20.12.2001)

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